FORM PTO-1390 (REV 1-98)

U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER

249-119P

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)

09/529937

INTERNATIONAL APPLICATION NO.	**INTERNATIONAL FILING DATE**	**PRIORITY DATE CLAIMED**
PCT/PT99/00015	August 17, 1999	August 21, 1998

TITLE OF INVENTION

DINITROANILINE LIPSOMAL FORMULATIONS AND PROCESSES FOR THEIR PREPARATION

APPLICANT(S) FOR DO/EO/US

MEIRINHOS DA CRUZ, Maria; CARVALHEIRO, Manuela; JORGE, Joao

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39 (1).
4. ☐ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau). WO 00/10532
   - b. ☒ has been transmitted by the International Bureau.
   - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(3)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(2)).
   - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   - b. ☐ have been transmitted by the International Bureau.
   - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.-1449 and International Search Report (PCT/ISA/210)
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:
    1.) Five (5) sheets of Formal Drawings

U.S. APPLICATION NO (if known, see 37 CFR 1 5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
NEW 09/529937	PCT/PT99/00015	249-119P

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO. . . . . **$970.00** International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO . . . . **$840.00** International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO. . . . . **$690.00** International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) . . . . **$670.00** International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4). **$96.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 970.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☒ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	20 - 20 =	0	X **$18.00**	$ 0	
Independent Claims	2 - 3 =	0	X **$78.00**	$ 0	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)		None	+ **$260.00**	$ 0	

TOTAL OF ABOVE CALCULATIONS =	$ 1100.00	
Reduction of ½ for filing by small entity, if applicable. Verified Small Entity statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).	$ 0	
SUBTOTAL =	$ 1100.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐20 ☐30 months from the earliest claimed priority date (37 CFR 1.492(f)). +	$ 0	
TOTAL NATIONAL FEE =	$ 1100.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +	$ 0	
TOTAL FEES ENCLOSED =	$ 1100.00	
	Amount to be: refunded	$
	charged	$

a. ☒ A check in the amount of $ 1100.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account. No. __________ in the amount of $_______ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-2448.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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PATENT
249-119P

IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicant: DA CRUZ, Maria et al.

Int'l. Appl. No.: PCT/PT99/00015

Appl. No.: New Group:

Filed: April 21, 2000 Examiner:

For: DINITROANILINE LIPOSOMAL FORMULATIONS AND PROCESSES FOR THEIR PREPARATION

PRELIMINARY AMENDMENT

BOX PATENT APPLICATION
Assistant Commissioner for Patents
Washington, DC 20231

April 21, 2000

Sir:

The following Preliminary Amendments and Remarks are respectfully submitted in connection with the above-identified application.

AMENDMENTS

IN THE SPECIFICATION:

Please amend the specification as follows:

Before line 1, insert --This application is the national phase under 35 U.S.C. § 371 of PCT International Application No. PCT/PT99/00015 which has an International filing date of August 17, 1999, which designated the United States of America.--

IN THE CLAIMS:

Please amend the claims as follows:

Claim 3: Line 1, change "any of the claims 1 and 2" to --claim 1--

Claim 4: Line 1, change "any of the claims 1 to 3" to --claim 1--

Claim 5: Line 1, change "any of the claims 1 to 4" to -- claim 1--

Claim 6: Line 1, change "any of the previous claims" to --claim 1--

Claim 10: Line 1, change "any of the claims 7 to 9" to --claim 7--

Claim 13: Line 1, change "any of the claims 7 to 12" to --claim 7--

Claim 15: Line 1, delete "according to claim 14,"

Claim 16: Line 1, delete "according to any of the claims 7 to 10 or 13 to 15,"

Claim 17: Line 1, delete "according to any of the claims 7 to 10 or 13 to 16,"

Claim 18: Line 1, delete "according to any of the claims 7 to 10 or 13 to 17,"

Claim 19: Line 1, change "any of the claims 7 to 18" to --claim 7--

Claim 20: Line 1, change "any of the claims 7 to 19" to --claim 7--

Claim 21: Line 1, change "any of the claims 1 to 6" to --claim 1--

Claim 22: Line 3, change "any of the claims 1 to 6 and 21" to --claim 1--

REMARKS

The specification has been amended to provide a cross-reference to the previously filed International Application. The claims have also been amended to delete the multiple dependencies and to place the application into better examination. Favorable action on the above-identified application is respectfully requested.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By ______________________
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249-119P

(Rev. 04/19/2000)

Description

DINITROANILINE LIPOSOMAL FORMULATIONS AND PROCESSES FOR THEIR PREPARATION

Field of Invention

This invention relates to liposomal compositions containing one or more dinitroanilines, incorporated or encapsulated, to processes for their preparation and to the use of these liposomal formulations in the treatment of infections in humans or animals.

The referred liposomal formulations can contain as dinitroaniline, for example, preferably, trifluralin (TFL).

Besides dinitroanilines, the liposomal formulations of the invention contain also phospholipids, individually or in mixtures, hydrogenated or not, with or without cholesterol (Chol) and electrically charged molecules, lipidic or not, as, for example, phosphatidylinositol (PI), phosphatidylglycerol (PG), dioleoylphosphatidylglycerol (DOPG), stearylamine (SA).

Invention background

The diseases caused by intracellular parasites of the mononuclear phagocytic system (MPS) cells are among the most important diseases all over the world due to the number of cases annually reported. One of these diseases is leishmaniasis, caused by a haemoflagellate protozoan named, in general, *Leishmania sp.* This disease has an incidence of at least 12 million infections in humans and animals. The dog has a crucial role as reservoir of the protozoan, being one among the responsible by the maintenance of zoonose. The disease is propagated from reservoirs to humans by vectors (sandflies). Leishmaniasis represents an immense public health problem in the Middle East, Africa, India,

China, Central and South America, and other tropical and subtropical areas throughout the world like the Mediterranean region including Portugal (33, 34).

Leishmaniasis, depending on the subspecies, can assume several forms of the disease: visceral, mucocutaneous and cutaneous. The visceral form of the disease is usually fatal if not treated. All forms may be linger and recurrent despite the treatment with pentavalent antimonial compounds, the recommended first choice drugs (20, 26, 33, 34).

Leishmania sp are able to live in the mononuclear phagocytic system cells, in an intracellular vesicle inside the host cell (2, 5, 14, 16, 31). The fusion of host cell lysosomes with the vacuole containing the parasite, does not prevent the *leishmania* multiplication. This fusion can even supply the necessary nutrients for its multiplication. In this way, the parasite seems to be safe inside the cell, being this one of the reasons why its elimination is so difficult. This fusion mechanism lysosome-vacuole can be used for alternative therapies namely through the internalisation mechanism of liposomes by MPS cells (2).

Several different classes of drugs have been used to treat leishmaniasis, namely pentavalent antimonial compounds, trivalent antimonial compounds, antibiotics (polyenics, aminoglucosides), immunomodulators (interferon α) and chelating agents (desferrioxiamine), among several others (26, 30, 32, 35).

The present recommended treatment for canine leishmaniasis is a course of pentavalent antimonial drugs, either sodium stibogluconate or meglumine antimoniate. These drugs have a limited effectiveness and they do not achieve a complete cure of the disease. These therapies are accompanied by a combination of problems, particularly: variable efficacy, long course of treatments and severe side effects such as cardiac and renal toxicities. Increased resistance to treatment with pentavalent antimonial drugs has also been reported and attributed to inadequate treatments (9, 20, 24, 26, 32, 33, 35).

Other drugs, used in the treatment of leishmaniasis, have limited clinic application also due to severe side effects, such as amphotericin B that is nephrotoxic (14, 20) and methotrexate (MTX) with cardiac toxicity (10, 11).

In spite some progresses in the development of new drugs have been achieved, none has 100% success in the treatment of the disease. Besides the referred toxic effects, the small efficacy of treatments is the other main disadvantage of the used drugs against infections by *Leishmania sp* (14).

As examples of these drugs it can be referred methotrexate (85% reduction on the infection level) (10, 11) pentamidine (less efficacy than antimonial derivatives), dehydroemetine, achieving 67% of cure (1) and desferrioxiamine, with 44% efficiency (32). In the cases of treatment failure with pentavalent antimonials, subsequent treatments with other classes of drugs, such as pentamidine, amphotericin B, ketoconazole and paramomycin, do not significantly increase the results. Also formycin B, sinefungin and lepidine WR6026 showed high antileishmanial activity when compared to pentavalent antimonials, but toxicity problems persist (26, 27).

Allopurinol and related compounds (allopurinol nucleoside, thiopurinol, thiopurinol ribonucleoside) have been tested *in vitro* and *in vivo* (27). The protozoans are not able to synthesise purines, being dependent on host purines and nucleosides. The presence of inosine analogues (e.g. allopurinol ribonucleoside) inhibits the purine metabolising enzymes of the parasite, affecting RNA function and reducing protein synthesis (26, 27). Previous studies showed that allopurinol, allopurinol nucleoside and thiopurinol ribonucleoside have small activity in animal models of the disease, probably due to the small residence time and low serum levels obtained by these drugs (27).

Antibiotics, such as, streptomycin and trobamycin inhibit the growth of both promastigote and amastigote forms of the parasite (25).

Trifluralin is a herbicide known to be active against leishmaniasis. This drug binds to plant tubulins but not to animal tubulins. *Leishmania sp* tubulins are very similar to plant tubulins. In this way, trifluralin showed to be able to inhibit promastigote proliferation, to reduce promastigote to amastigote transformation, to interfere with amastigote replication and to reduce amastigote infectivity. *In vitro*

studies confirmed efficiency against all forms of leishmaniasis, but *in vivo* studies only presented good results against the cutaneous forms of the disease. A drug delivery system need to be developed for the use of trifluralin against visceral forms since trifluralin solubility and lipophilicity do not allow the administration by any other route than the topical one. By this route no activity against the visceral forms was observed (12).

In view of the difficulties above described, an alternative approach to the search of new drugs is the drug encapsulation in macrophage directed carriers, as liposomes.

Liposomes are phospholipid synthetic bilayer vesicles able of incorporating a variety of substances independently of their molecular weight, electrical charge and solubility (18, 19, 23).

The rationale for the use of liposomal associated drugs instead of free drugs for the treatment of visceral leishmaniasis rely on the fact that amastigotes of the parasite are specifically located in liver spleen and bone marrow macrophages. As liposomes are preferentially taken up by these cells (2, 5, 30), they can deliver toxic agents straight to the intracellular location of established parasites (28). Though, the administration of liposome-encapsulated agents theoretically increases the therapeutic index of the agent in two ways: 1) increasing the uptake of the carrier and consequently of the drug by macrophages contained organisms, and 2) reducing toxicity of the free drug due to relatively low uptake of carrier by organs to which the drug is toxic (2, 6, 16, 20).

The great majority of drug delivery systems administered by intravenous route are taken up from circulation by the liver, meaning that they accumulate preferentially in this organ, not achieving, at significant quantities, other organs also belonging to MPS (spleen and bone marrow). The uptake by these other organs can be increased by the reduction on vesicle diameter (8). This can result in the suppression of the infection in the spleen and bone marrow, quite difficult to achieve with the free drugs.

Results in literature show that liposome encapsulated drugs are much safer and more effective to treat MPS infection as compared to free drugs (5, 17, 28).

Liposomal formulations of pentavalent antimonials can increase 200 to 700 times the therapeutic index compared with the free form, depending upon the lipid composition of liposomes (4, 7, 8, 13). Liposomal amphotericin B is 2 to 5 times more active than free form (6, 29). Liposomal primaquine presents activity at doses not actives for the free form (3). However these results are strongly dependent from the infection stage at the beginning of treatment and are difficult to correlate due to the heterogeneity of the experimental conditions (15).

Most of the above described results suffer from limitations of the kind of liposomes used, with low encapsulation efficiency and of small half lives, not reaching crucial targets for the treatment of this disease and, also, because of high costs. Liposomal amphotericin B was effective in curing a few cases of visceral leishmaniasis in humans (16, 21, 22), but the cost of such a treatment is too high for widely application to animals infected population.

Invention Detailed Description

The present invention refers to liposomal formulations containing one or several dinitroanilines e to processes for their preparation.

The present invention concerns the achievement, under stable form, lyophilised or not, of liposomal formulations containing one or several dinitroanilines, for example trifluralin incorporated or encapsulated.

In the formulations obtained according to the present invention, the liposomal diameter varies between 0,01 μm to 50 μm. According to one of the preferred forms of preparation, mixtures of different size populations exist in the formulations of the present invention, with diameters respectively bigger and lower than 100 nm in a specially preferred form.

Additionally the present invention formulations may contain any of the following lipids, hydrogenated or not, individually or in mixtures, in any molar ratio: distearoylphosphatidylcholine (DSPC), phosphatidylcholine (PC), cholesterol (Chol) or derivatives, sphingomielin (SM), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), phosphatidylglycerol (PG), dimiristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC),

gangliosides, ceramides, phosphatidylinositol (PI), phosphatydic acid (PA), dicetylphosphate (DcP), dimiristoylphosphatidylglycerol, (DMPG), stearylamine (SA), dipalmitoylphosphatidylglycerol (DPPG) and other synthetic lipids.

The preparation process of the present invention liposomal formulations comprises the steps of:

– hydration from a lipid film containing the dinitroaniline for the achievement of a liposomal formulation

– lyophilization

– rehydration

In a common way, solubilization in organic solvent of the lipidic components and the dinitroaniline or dinitroanilines, for example trifluralin, can be done, followed by drying under N_2 stream or under vacuum, for example, in a rotavapor with controlled temperature for the achievement of a mixed homogeneous film of lipid and dinitroaniline or dinitroanilines, for example, trifluralin. This film can be, subsequently, hydrated with a sugar solution forming multillamelar liposomes. The following step can be the liposomal formulation sizing, under pressure, by successive extrusions through polycarbonate membranes of pore sizes varying from 5,0 to 0,01 µm. The sizing will end preferably after extrusion through the membrane with the desired pore size for a part of the population. After the attainment of the necessary different populations with well-determined diameter, the following step is the mixture of these populations.

After the attainment of the necessary different populations of well-determined diameter, the following step is the mixture of these populations. After the mixture of the populations, it can be, or not, done a concentrative dialysis, using, for example, polyethyleneglycol as hygroscopic agent, followed by a step of dehydration. This dehydration occurs preferably in the presence of sugars that will act as protective of sublimation of the dinitroaniline or dinitroanilines, for example, trifluralin.

The formulations according the present invention so obtained, after hydration with water, are ready for use.

Up to now, there is no literature reference to liposomal preparations with dinitroanilines.

According the present invention, in order to prepare the multillamelar liposomes a step of drying a mixture of one dinitroaniline, namely trifluralin, and lipids, both solubilized in the same solvent or mixture of organic solvents, is performed. The amount of trifluralin varies according the final volume to prepare, ranging from 10 µg to 1 g or more. The amount of lipid also changes according the final volume to be prepared, ranging from 1 µmole to 1 mole or more. The adequate lipids, hydrogenated or not for the preparation of the formulations are present individually or in mixtures, in any molar ratio from the following lipids: distearoylphosphatidylcholine (DSPC), phosphatidylcholine (PC), cholesterol (Chol) or derivatives, sphingomielin (SM), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), phosphatidylglycerol (PG), dimiristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), gangliosides, ceramides, phosphatidylinositol (PI), phosphatydic acid (PA), dicetylphosphate (DcP), dimiristoylphosphatidylglycerol, (DMPG), stearylamine (SA), dipalmitoylphosphatidylglycerol (DPPG) and other synthetic lipids.

The so obtained mixture is submitted to a step of drying under a N_2 stream, until the total remove of the solvent or mixture of solvents. After drying, hydration of the mixture with a solution of a sugar as, for example, trehalose, is done, ranging its concentration from 0,01 M to 2 M, under mechanical stirring or manual external stirring. The so obtained liposomal formulation is, then, submitted to a step of sizing, for example, by successive passages under pressure through polycarbonate filters of decreasing pore diameter, normally referred as extrusion. Extrusion starts normally through 5 µm diameter pore membranes and continues with passages through diameter pore membranes of 2, 1, 0,8, 0,6, 0,4, 0,2, 0,1 and 0,05 µm, reaching some times 0,02 µm. According to a preferred way preparation of the invention and after passage through 0,4 µm membranes, the so obtained liposomal preparation is split in two parts. Only one of those parts goes trough the rest of the extrusion procedure until, for example, 0,05 µm diameter

pore membranes. At the end, the two parts that correspond to two distinct populations are mixed, achieving, by this way, one liposomal formulation containing liposomes that exhibit two different diameter distribution populations. The simultaneous presence of these different diameter populations present the advantage that, after *in vivo* parenteral administration, the population of bigger diameter is rapidly captured by mononuclear phagocytic system cells, while the small size population remains in circulation, possibly reaching organs other than liver and spleen, where the parasitic infection also exist, as for example, the bone marrow.

The so obtained formulation may be, or not, submitted to a step of concentration by dialysis against, for example, polyethyleneglycol that will act as a water removing agent. After this dialysis step, the formulation can be frozen up to –70°C during, at least one hour, after what it is submitted to lyophilization

After this lyophilization, the formulation is ready to be used, being enough for that, the addiction of water to the so obtained powder. Hydration occurs instantaneously originating one homogeneous suspension in water of liposomes, containing the dinitroaniline or dinitroanilines as, for example, trifluralin.

A particularly preferred way of preparation of the present invention is the one in which to a lipid mixture of DOPC:DOPG in a molar ratio of 7 : 3, in a total of 10 μmole of lipid, solubilized in chloroform, is added 1 μmole of trifluralin, solubilized in chloroform. The obtained mixture is, then, dried under a stream of nitrogen until total evaporation of the chloroform. The so film is hydrated with 0,1 mL of 0,3 M trehalose, with manual stirring. After complete resuspension of the lipidic film, the formulation rests for 15 minutes, after what 0,1 mL more of the same trehalose solution is added. Another 15 minutes resting period is allowed after what hydration is completed by adding 0,8 mL of the same solution. The so obtained liposomal formulation is submitted to a sizing step by passage under pressure through polycarbonate membranes of successively decreasing pore diameters, from 5 μm to 0,4 μm. After this extrusion procedure, the formulation is

divided in two equal parts. One of those parts continues the sizing step until a filter of 0,05 μm. The two populations so obtained are, then, mixed. The mixed liposomal formulation is submitted to freezing at –70°C for 60 minutes and, after that period, lyophilised. In this way, a liposomal formulation ready to be hydrated with 1,0 mL of distilled sterile water is obtained, able to be parenterically administered.

The formulations may also contain auxiliary substances, pharmaceutically acceptable, useful for preservation of their quality and or to turn them closely related to physiological conditions, such as pH adjusting agents, buffering agents, tonicity agents, antioxidants and other adjuvants as, for example, sodium acetate, sodium lactate, sodium chloride, potassium, chloride, calcium chloride, glucose, saccharose, mannitol, xylitol, alpha-tocopherol.

The pharmaceutical formulations obtained according the present invention can be administered to warm blood animals, such as man, already suffering from leishmaniasis, during the necessary time interval and in a necessary quantity to end or significantly inhibit infection progress. The adequate quantities for the achievement of that effect are named as "therapeutically efficient doses". The therapeutic efficient doses for this use will depend on the infection degree and on the general state of health of the treatment individual. There is no other formulation of the free drug, namely, trifluralin, used in parenteric administration.

The following examples, of liposomal formulations prepared according the present invention and of their respective physico-chemical and biological analysis, are presented as illustrations and not as limitations.

Literature

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Examples

These examples illustrate liposomal formulation prepared according to the present invention and processes for their preparation in which the used dinitroaniline is trifluralin.

Trifluralin (TFL) incorporation in liposomes

The preparation of the following described formulations, in a total volume of 5 mL for lipidic composition, started by the addition of TFL to lipid in chloroform, followed by evaporation of the solvent under nitrogen stream. Hydration of the resulting film was done by adding 500 µL of trehalose 0,3 M, stirring and resting for 15 minutes, addition of 500 µL more of 0,3 M trehalose, stirring again and resting again for a new 15 minute period and, finally, by the addition of 4000 µL of 0,3 M trehalose. Samples for dosage (initial TFL and initial lipid) were removed. The liposomal formulations so obtained were sized by successive filtration, under nitrogen pressure, through polycarbonate filters with pores of 5,0, 2,0, 1,0, 0,8, 0,6 and 0,4 µm, with two passages in the last filter (extrusion). The non-incorporated TFL, as it is insoluble in aqueous solutions, crystallises on a needle type structure and remains at the top of the filters. After extrusion through 0,4 µm filter, the formulations are split in two equal parts. With one of those part extrusion procedure continues, now through diameter pore membranes of 0,2 and 0,1 µm, with two passages in the last filter. The liposomal formulation half part that was extruded until membranes of 0,4 µm pore diameter, is named VET400 (Vesicles Extruded Through 400 nm). The liposomal formulation half part that was extruded until membranes of 0,1 µm pore diameter, is named VET400 (Vesicles Extruded Through 100 nm). The VET400 and VET100 formulations obtained by the previous process are finally submitted to dosage (final TFL and final LIP).

Table 1a represents the lipid composition effect on the incorporation parameters of liposomes sized until 0,4 µm pore filters. **Table 1b** represents the lipid

composition effect on the incorporation parameters of liposomes sized until 0,1 µm pore filters. The formulations were prepared with different lipid compositions, with lipid and TFL quantities presented in the referred tables. Incorporation efficiency (I.E.) represent the ratio between the final (drug to lipid ratio) and the initial (drug to lipid ratio) and is expressed as a percentage. The recovery is also expressed as a percentage and can be referred to drug (TFLf/TFLi) or to lipid (LIPf/LIPi).

TABLE 1a - Lipid composition effect on incorporation parameters of TFL in liposomes sized until 0.4 µm

Formulation number	Lipidic composition Molar Ratio		TFLi (µg)	TFLf (µg)	LIPi (µmol)	LIPf (µmol)	(TFL/LIP)i (g/mol)	(TFL/LIP)f (g/mol)	LIPf/LIPi (%)	TFLf/TFLi (%)	I.E. (%)
1	PC:CHOL 2:1	average	332,03	140,78	11,52	9,48	29,09	14,37	81,72	44,89	52,97
		standard deviation	43,50	75,14	0,82	2,24	5,80	5,65	13,62	28,53	27,54
		median	330,73	144,47	11,41	8,25	28,99	17,52	75,55	43,68	60,43
2	DMPC:CHOL 2:1	average	367,60	110,90	12,58	10,20	30,02	11,30	80,37	30,31	37,95
		standard deviation	50,82	14,34	3,02	3,00	5,71	2,16	6,08	3,55	6,17
		median	346,53	119,18	13,87	10,75	28,85	11,08	77,52	28,53	37,42
3	DSPC:CHOL 2:1	average	390,66	170,81	13,48	10,75	29,13	15,88	79,86	44,09	55,24
		standard deviation	64,63	29,75	1,33	1,03	5,04	2,37	4,10	8,05	9,76
		median	358,40	185,56	13,76	10,35	29,79	15,56	81,42	40,92	54,40
4	DPPC:CHOL 2:1	average	322,83	84,72	9,14	7,38	35,37	11,17	80,88	24,75	31,59
		standard deviation	31,36	82,60	0,98	0,87	2,14	10,17	6,80	23,86	29,64
		median	334,68	89,15	8,85	7,31	34,43	13,61	80,99	26,64	35,99
5	HPC:CHOL 2:1	average	328,77	128,65	10,34	8,13	36,97	16,55	81,90	39,41	49,77
		standard deviation	24,77	11,07	3,92	2,32	20,48	3,77	14,78	5,79	14,16
		median	330,73	133,40	12,46	8,45	26,54	16,17	80,69	41,29	54,55
6	PC:CHOL 4:1	average	309,65	190,30	10,40	8,49	29,78	22,42	81,61	61,42	75,26
		standard deviation	8,22	11,06	0,23	0,10	1,37	1,12	2,40	2,06	0,62
		median	307,02	190,30	10,30	8,45	29,81	22,63	81,65	61,98	75,19
7	HPC:CHOL 4:1	average	334,68	114,96	11,87	8,49	29,71	13,29	71,63	35,35	50,85
		standard deviation	47,42	52,36	2,77	2,46	10,81	3,61	11,53	16,98	25,27
		median	334,68	109,70	11,95	7,16	24,03	14,99	77,52	38,19	65,43
8	PC:PG 4:1	average	318,87	323,03	11,85	11,90	26,90	27,15	100,40	101,34	100,93
		standard deviation	3,95	8,51	0,16	0,12	0,19	0,99	0,43	3,85	3,59
		median	318,90	326,70	11,76	11,86	26,81	27,55	100,34	102,45	101,58
9	DMPC:CHOL 4:1	average	388,04	117,60	12,19	9,56	31,98	12,37	78,46	30,27	38,63
		standard deviation	69,86	22,30	2,40	1,87	2,42	1,24	4,06	0,70	1,59
		median	370,24	114,44	12,60	10,46	32,37	13,06	77,03	30,38	38,32
10	DSPC:CHOL 4:1	average	325,36	135,52	11,76	9,19	28,87	15,22	79,99	42,08	52,47
		standard deviation	41,59	12,68	3,52	1,78	6,17	3,72	9,53	6,33	1,76
		median	336,29	136,60	11,99	9,29	30,06	15,89	77,48	40,96	52,87

The presented values are the average, standard deviation and median of, at least, three preparations

Abbreviations:

TFLi Inicial trifluralin

TFLf Final trifluralin

LIPi Initial lipid

LIPf Final lipid

(TFL/LIP)i Initial ratio TFL/LIP

(TFL/LIP)f Initial ratio TFL/LIP

LIPf/LIPi LIP recovery

TFLf/TFLi TFL recovery

I.E. Incorporation efficiency

TABLE 1a - Lipid composition effect on incorporation parameters of TFL in liposomes sized until 0.4 μm (cont.)

Formulation number	Lipidic composition Molar Ratio		TFLi (μg)	TFLf (μg)	LIPi (μmol)	LIPf (μmol)	(TFL/LIP)i (g/mol)	(TFL/LIP)f (g/mol)	LIPf/LIPi (%)	TFLf/TFLi (%)	I.E. (%)
11	DPPC:CHOL	average	318,22	82,83	9,11	7,82	34,72	10,26	86,15	24,39	28,97
	4:1	standard deviation	64,51	55,90	1,22	0,79	2,47	6,43	5,17	14,06	17,36
		median	310,97	97,05	9,18	7,52	33,86	12,90	84,64	31,21	38,10
12	PC:CHOL (Soy bean)	average	397,89	165,01	10,45	7,68	39,69	21,68	73,84	41,25	56,09
	4:1	standard deviation	22,78	32,43	2,63	1,83	9,65	1,37	5,23	5,84	9,00
		median	395,90	166,59	11,93	8,09	35,10	21,23	76,62	42,08	60,49
13	DPPC:DPPG	average	301,09	101,27	9,49	6,12	31,76	16,70	64,29	33,63	52,49
	7:3	standard deviation	13,69	7,80	0,50	0,98	1,80	1,69	7,18	2,16	2,47
		median	293,19	104,95	9,67	6,12	32,76	17,41	63,26	33,62	53,14
14	DOPC:DOPG	average	323,50	178,09	10,35	8,17	31,31	22,04	78,27	55,36	71,36
	7:3	standard deviation	37,57	33,67	0,80	2,05	3,40	2,14	13,64	10,93	14,41
		median	324,80	185,56	10,37	7,67	32,35	22,02	73,99	57,55	64,66
15	PC:CHOL:PG	average	313,63	148,11	12,33	10,44	25,69	13,86	85,09	46,77	55,25
	10:5:1	standard deviation	44,63	58,94	2,19	1,50	3,48	3,71	5,56	14,64	18,66
		median	309,00	139,70	11,82	10,75	24,48	13,00	84,43	51,45	56,58
16	PC:CHOL:PI	average	337,98	143,42	12,23	9,45	27,98	15,17	76,72	41,89	54,68
	10:5:1	standard deviation	40,38	42,01	2,55	2,67	2,73	0,72	8,44	7,00	7,22
		median	320,85	120,76	11,13	8,40	27,76	15,38	80,69	38,06	56,78
17	PC:CHOL:SA	average	290,55	95,05	10,54	8,32	27,79	10,72	77,60	31,24	38,37
	10:5:1	standard deviation	35,45	81,33	1,89	2,85	2,09	6,87	12,71	23,70	25,01
		median	277,39	81,25	10,38	7,39	26,72	13,42	71,16	30,83	44,43
18	DPPC:CHOL:DPPG	average	324,80	23,82	9,03	6,93	35,87	3,36	76,96	7,53	9,42
	10:5:1	standard deviation	47,95	10,52	0,26	1,26	4,34	1,24	16,02	3,71	3,38
		median	338,63	29,09	9,15	6,82	37,01	3,53	73,96	8,42	11,37
19	PC:CHOL:DSPE-PEG	average	231,29	213,26	9,47	9,01	24,41	23,69	95,12	92,39	97,10
	3.7:1:0.3	standard deviation	13,16	3,65	0,06	0,28	1,26	0,97	3,51	5,15	2,95
		median	227,99	215,37	9,46	9,12	24,20	23,36	96,82	91,69	96,20
20	DSPC:CHOL:DSPE-PEG	average	322,82	192,21	10,20	8,26	32,73	22,96	80,10	64,79	76,58
	8:2:0.5	standard deviation	60,28	85,16	1,68	2,61	10,23	5,35	14,25	42,28	36,40
		median	350,48	171,01	10,45	7,11	34,86	25,42	76,43	48,79	63,84

The presented values are the average, standard deviation and median of, at least, three preparations

Abbreviations:

TFLi Inicial trifluralin
TFLf Final trifluralin
LIPi Initial lipid
LIPf Final lipid
(TFL/LIP)i Initial ratio TFL/LIP
(TFL/LIP)f Initial ratio TFL/LIP
LIPf/LIPi LIP recovery
TFLf/TFLi TFL recovery
I.E. Incorporation efficiency

TABLE 1b - Lipid composition effect on incorporation parameters of TFL in liposomes sized until 0.1 μm

Formulation number	Lipidic composition Molar Ratio		TFLi (μg)	TFLf (μg)	LIPi (μmol)	LIPf (μmol)	(TFL/LIP)i (g/mol)	(TFL/LIP)f (g/mol)	LIPf/LIPi (%)	TFLf/TFLi (%)	I.E. (%)
1	PC:CHOL 2:1	average	332,03	70,67	11,52	7,15	29,09	9,47	61,60	22,54	34,74
		standard deviation	43,50	42,29	0,82	2,39	5,80	2,71	17,24	16,29	16,65
		median	330,73	46,79	11,41	6,68	28,99	9,29	62,05	14,15	32,06
2	DMPC:CHOL 2:1	average	367,60	48,20	12,58	8,02	30,02	6,56	64,07	13,32	20,74
		standard deviation	50,82	18,51	3,02	1,73	5,71	3,96	3,22	5,94	8,80
		median	346,53	49,95	13,87	8,90	28,85	5,61	65,81	11,74	19,45
3	DSPC:CHOL 2:1	average	390,66	94,90	13,48	8,18	29,13	12,09	61,43	24,80	43,97
		standard deviation	64,63	10,82	1,33	1,60	5,04	3,87	15,02	5,48	22,33
		median	358,40	92,10	13,76	8,45	29,79	10,15	69,97	23,93	34,07
4	DPPC:CHOL 2:1	average	322,83	65,26	9,14	6,46	35,37	8,35	69,94	18,93	24,09
		standard deviation	31,36	88,39	0,98	1,77	2,14	10,04	11,69	25,45	29,91
		median	334,68	29,93	8,85	5,49	34,43	5,55	65,79	8,94	14,69
5	HPC:CHOL 2:1	average	328,77	100,88	10,34	8,06	36,97	13,55	79,50	30,89	39,15
		standard deviation	24,77	6,17	3,92	2,64	20,48	4,81	6,53	4,26	7,08
		median	330,73	98,42	12,46	9,19	26,54	10,85	78,13	29,76	40,34
6	PC:CHOL 4:1	average	309,65	114,22	10,40	6,18	29,78	18,48	59,42	36,89	62,12
		standard deviation	8,22	3,80	0,23	0,07	1,37	0,40	1,43	1,11	2,89
		median	307,02	115,28	10,30	6,20	29,81	18,59	59,22	36,81	60,50
7	HPC:CHOL 4:1	average	334,68	73,83	11,87	7,42	29,71	9,72	62,43	22,60	36,47
		standard deviation	47,42	30,47	2,77	2,00	10,81	1,63	6,04	9,88	15,63
		median	334,68	67,86	11,95	6,63	24,03	10,23	65,45	23,62	42,58
8	PC:PG 4:1	average	318,87	324,60	11,85	12,45	26,90	26,08	105,06	101,82	96,95
		standard deviation	3,95	2,97	0,16	0,32	0,19	0,86	2,34	2,15	3,51
		median	318,90	325,90	11,76	12,63	26,81	25,80	105,07	102,20	95,16
9	DMPC:CHOL 4:1	average	388,04	52,41	12,19	7,22	31,98	6,96	57,76	13,11	21,95
		standard deviation	69,86	26,11	2,40	2,74	2,42	1,29	14,36	5,49	4,90
		median	370,24	63,65	12,60	8,75	32,37	7,19	60,88	15,27	24,45
10	DSPC:CHOL 4:1	average	335,36	87,53	11,76	7,98	29,53	11,01	69,57	25,93	37,51
		standard deviation	48,56	26,75	3,52	1,48	5,12	2,82	8,57	5,82	8,60
		median	360,40	79,45	11,99	8,32	30,06	10,34	69,39	23,53	35,51

The presented values are the average, standard deviation and median of, at least, three preparations

Abbreviations:

TFLi Inicial trifluralin
TFLf Final trifluralin
LIPi Initial lipid
LIPf Final lipid
(TFL/LIP)i Initial ratio TFL/LIP
(TFL/LIP)f Initial ratio TFL/LIP
LIPf/LIPi LIP recovery
TFLf/TFLi TFL recovery
I.E. Incorporation efficiency

TABLE 1b - Lipid composition effect on incorporation parameters of TFL in liposomes sized until 0.1 μm (cont.)

Formulation number	Lipidic composition Molar Ratio		TFLi (μg)	TFLf (μg)	LIPi (μmol)	LIPf (μmol)	(TFL/LIP)i (g/mol)	(TFL/LIP)f (g/mol)	LIPf/LIPi (%)	TFLf/TFLi (%)	I.E. (%)
11	DPPC:CHOL	average	318,22	58,03	9,11	6,29	34,72	8,85	69,09	16,84	24,93
	4:1	standard deviation	64,51	44,95	1,22	1,50	2,47	5,36	13,59	10,32	14,02
		median	310,97	48,90	9,18	6,07	33,86	9,97	76,57	15,72	29,44
12	PC:CHOL (Soya been)	average	397,89	109,66	10,45	5,80	39,69	18,89	56,55	27,45	49,54
	4:1	standard deviation	22,78	19,83	2,63	0,96	9,65	0,84	6,92	3,82	12,22
		median	395,90	119,49	11,93	6,02	35,10	18,53	55,12	29,09	52,78
13	DPPC:DPPG	average	305,04	33,09	8,71	3,83	35,03	8,83	44,42	10,90	25,40
	7:3	standard deviation	25,83	12,77	0,32	1,65	2,54	0,80	20,32	4,48	4,00
		median	293,19	35,20	8,85	4,30	34,43	8,59	48,59	10,52	24,95
14	DOPC:DOPG	average	323,50	109,74	10,35	5,48	31,31	19,61	52,13	33,51	63,77
	7:3	standard deviation	37,57	54,44	0,80	2,32	3,40	3,19	18,10	14,10	16,65
		median	324,80	100,53	10,37	4,55	32,35	20,71	43,88	35,24	64,03
15	PC:CHOL:PG	average	313,63	106,14	12,33	8,91	25,69	11,82	73,21	33,41	47,11
	10:5:1	standard deviation	44,63	45,41	2,19	1,21	3,48	4,64	11,70	11,11	20,89
		median	309,00	97,36	11,82	9,06	24,48	9,70	73,14	35,86	42,22
16	PC:CHOL:PI	average	337,98	111,78	12,23	7,86	27,98	13,36	62,87	31,73	48,78
	10:5:1	standard deviation	40,38	74,27	2,55	3,14	2,73	3,43	12,67	17,06	17,14
		median	320,85	71,02	11,13	6,54	27,76	11,95	62,82	22,13	43,05
17	PC:CHOL:SA	average	290,55	40,34	10,54	6,42	27,79	7,87	57,74	13,51	27,38
	10:5:1	standard deviation	35,45	26,53	1,89	3,96	2,09	6,49	26,86	7,79	20,76
		median	277,39	40,47	10,38	5,99	26,72	6,31	57,67	15,35	23,87
18	DPPC:CHOL:DPPG	average	324,80	14,83	9,03	4,39	35,87	6,58	48,86	4,36	17,71
	10:5:1	standard deviation	47,95	8,84	0,26	2,67	4,34	7,90	30,20	2,32	21,32
		median	338,63	19,39	9,15	5,69	37,01	3,41	61,73	5,32	8,62
19	PC:CHOL:DSPE-PEG	average	231,29	188,33	9,47	9,09	24,41	20,68	96,02	81,90	84,91
	3.7:1:0.3	standard deviation	13,16	34,07	0,06	1,48	1,26	0,46	16,20	17,32	5,97
		median	227,99	182,71	9,46	8,75	24,20	20,88	92,49	83,01	86,75
20	DSPC:CHOL:DSPE-PEG	average	322,82	159,37	10,20	7,22	32,73	25,65	69,44	50,96	76,60
	8:2:0.5	standard deviation	60,28	30,66	1,68	3,99	10,23	10,21	29,97	15,04	11,23
		median	350,48	163,74	10,45	5,73	34,86	30,25	68,21	53,53	78,47

The presented values are the average, standard deviation and median of, at least, three preparations.

Abbreviations:

TFLi Inicial trifluralin
TFLf Final trifluralin
LIPi Initial lipid
LIPf Final lipid
(TFL/LIP)i Initial ratio TFL/LIP
(TFL/LIP)f Initial ratio TFL/LIP
LIPf/LIPi LIP recovery
TFLf/TFLi TFL recovery
I.E. Incorporation efficiency

From the analysis of **Table 1a** and **1b** important conclusion can be drawn, referring to the effect of the presence of cholesterol in lipid composition, the effect of electrically charged molecules in lipid composition and to the effect of lipids with different phase transition temperature. The obtained results evidence that TFL is better incorporated in liposomes with low content of cholesterol, as can be observed in **Figure 1** (in the formulation composed of PC:CHOL in a molar ratio of 4:1). The most significative difference was observed to the small size liposomes (VET100). In what concerns the presence of electrically charged molecules in the lipid composition it can be concluded that TFL is poorly incorporated in positively charged liposomes (PC:CHOL:SA) and that, in spite of the better results had been obtained with electrically neutral liposomes (PC:CHOL), negatively charged liposomes (PC:CHOL:PI and PC:CHOL:PG) present good values of incorporation, as can be seen in **Figure 2**. This is an important result, as it is known that negatively charged liposomes, after parenterical administration, have longer circulating times. In what concerns the use of lipids with different phase transition temperature, a comparison can be made between the results obtained for the liposomal formulations with PC, HPC and DSPC (increasing phase transition temperature) in different proportions with CHOL. For the formulations with lower proportion of cholesterol, the lipid with lower transition phase temperature (PC) revealed the best results for TFL incorporation. However, when the cholesterol proportion increases, the lipid with bigger transition phase temperature (DSPC) revealed the best results, even though the difference is not significative, as can be observed in **Figure 3**.

In vitro stability of two trifluralin liposomal formulations

The preparation of the liposomal formulations for this stability study *in vitro*, 4 mL initial volume containing 10 µmole/mL of lipid (PC:PG) in 4:1 molar ratio and 1 µmole/mL (335 µg/mL) of TFL, started by the addition of TFL to the lipid in chloroform, followed by evaporation of the solvent under nitrogen stream. The hydration of the resulting film was carried out by addition of 400 µL of 0,3 M

trehalose, stirring, 15 minute rest, addition of 400 µL more of 0,3 M trehalose, stirring again and resting again for more 15 minute and, finally, with the addition of 3200 µL of 0,3 M trehalose.

The so obtained liposomal formulations were sized by successive filtration under nitrogen pressure, through polycarbonate filters with pores of 5,0, 2,0, 1,0, 0,8, 0,6 and 0,4 µm, with two passages in this late filter (extrusion). The non-incorporated TFL, as it is insoluble in aqueous solutions, crystallises on a needle type structure and remains at the top of the filters. After extrusion through 0,4 µm filter, the formulations are split in two equal parts. With one of those parts extrusion procedure continues, now through diameter pore membranes of 0,2 and 0,1 µm, with two passages in the last filter. The two formulations obtained according to the previous process were kept at 4°C and samples for dosage of TFL were removed at days 0, 1, 2, 3, 4, 6, 8 and 10, being the result expressed by comparison with the value obtained for day O, in percentage. Immediately before the sampling, the formulations were microscopically observed for crystal detection that, if present, would be removed by centrifugation.

As can be seen in **Figure 4**, the two liposomal formulations (VET 400 and VET100) are stable, upon hydration, presenting 100% stability in the first 48 hours. The experience was repeated three times, being the presented values, the median of the results obtained for each time point.

Stability on storage of three different trifluralin liposomal formulations

The preparation of the liposomal formulations for this stability study *in vitro*, 45 mL initial volume containing 10 µmole/mL of lipid (DOPC:DOPG) in 7:3 molar ratio and 1 µmole/mL (335 µg/mL) of TFL, started by the addition of TFL to the lipid in chloroform, followed by evaporation of the solvent under nitrogen stream. The hydration of the resulting film was carried out by addition of 4,5 mL of 0,3 M trehalose, stirring, 30 minute rest, addition of 4,5 mL more of 0,3 M trehalose, stirring again and resting again for more 15 minute and, finally, with the addition of 36 mL of 0,3 M trehalose.

The so obtained liposomal formulations were sized by successive filtration under nitrogen pressure, through polycarbonate filters with pores of 5,0, 2,0, 1,0, 0,8, 0,6 and 0,4 µm, with two passages in this late filter (extrusion). The non-incorporated TFL, as it is insoluble in aqueous solutions, crystallises on a needle type structure and remains at the top of the filters. After extrusion through 0,4 µm filter, the formulations are split in two equal parts. With one of those parts extrusion procedure continues, now through diameter pore membranes of 0,2 and 0,1 µm, with two passages in the last filter. From the two formulations VET400 and VET 100 obtained according to the previous process, equal amounts were taken and mixed, being this mixture of the two previous formulations named as MIX liposomal formulation. The three liposomal formulations were then split by vials of 1 mL each, frozen at –70°C during 1 hour and lyophilised overnight. After the lyophilization procedure the vials were closed, under vacuum, sealed with aluminium caps and placed in the benchtop for the entire time of the stability study. For each experimental point (0, 0,03, 0,07, 0,13, 0,23, 0,47, 0,7, 1, 2, 3, 4, 5, 6 and 12 months), vials were opened, hydrated with deionised sterile water until the final volume of 1 mL. The vials containing the liposomal formulations were left to stand for 2 hours. The formulations were microscopically observed for crystal detection that, if present, would be removed by centrifugation. Quantification of TFL was carried out for each formulation and the results expressed as the percentage of TFL as compared to day 0 (final day of lyophilization).

As can be seen from Figure 5, the three liposomal formulations (VET 400, VET100 and MIX) are stable, in the lyophilised form, presenting, after one year of preparation followed by water hydration, TFL values bigger than 90% of the initial value. The experiment was conducted in triplicate and the presented values represent the median of the obtained values for each point.

Single dose toxicity evaluation

This study was performed with a liposomal formulation of TFL with DOPC:DOPG 7:3 as the lipid composition and compared with a liposomal formulation with equal lipid composition without TFL (empty liposomes).

The preparation of the empty liposomal formulation for this single dose toxicity study, 400 mL initial volume containing 10 μmole/mL of lipid (DOPC:DOPG) in 7:3 molar ratio, started by measuring of lipid in chloroform, followed by evaporation of the solvent under nitrogen stream. The hydration of the resulting film was carried out by addition of 40 mL of 0,3 M sucrose, stirring, 30 minute rest, addition of 40 mL more of 0,3 M sucrose, stirring again and resting again for more 15 minute and, finally, with the addition of 320 mL of 0,3 M sucrose.

The so obtained empty liposomal formulation was sized by successive filtration under nitrogen pressure, through polycarbonate filters with pores of 5,0, 2,0, 1,0, 0,8, 0,6 and 0,4 μm, with two passages in this late filter (extrusion). After extrusion the liposomal formulation was submitted to ultracentrifugation at 49.000 rpm, for two hours, at 15°C. After ultracentrifugation, supernatant was removed and the pellet was ressuspended until 35 mL by addition of 0,3 M sucrose.

The preparation of the TFL liposomal formulation was performed according to the same process described for the empty formulation, with TFL being added to the initial solution of lipid in chloroform. After extrusion through 0,4 μm filter, the formulations are split in two equal parts. With one of those parts extrusion procedure continues, now through diameter pore membranes of 0,2 and 0,1 μm, with two passages in the last filter. The TFL formulations VET400 and VET100 obtained according to the previous process were mixed, being this mixture of the two previous formulations named as MIX liposomal formulation. The formulation was microscopically observed for crystal detection that, if present, would be removed by centrifugation.

The final lipid concentration was determined in both empty and TFL liposomal formulations being the late one adjusted in a way that both formulations

contained exactly the same concentration of lipid. After this adjustment the TFL concentration was determined in the TFL containing formulation.

The study was carried out in BALB/c male and female mice. The liposomal formulations were administered by two routes: intraperitoneal (i.p.) and intravenous (i.v.). The administered doses were 30, 20 and 10 mL/kg for the i.p. route and of 10, 5 and 2 mL/kg for the i.v. route of administration. 5 animal per group were used. The administered doses correspond to calculated doses of lipid (in both formulations) and of TFL (in the TFL containing formulation) presented in **Table 2.**

Table 2 – Single dose toxicity doses

Dose (mL/Kg)	Lipid (µmole/Kg)	TFL (mg/Kg)
30	2790	63.9
20	1860	42.6
10	930	21.3
5	465	10.7
2	186	4.3

All animals were weighted and the amount of liposomal formulation was calculated according to the measured weight in order to achieve the desired dose, in mL/kg. One animal group per sex was injected with 0,3 M sucrose as control group

The animals were observed at regular intervals, during 48 hours after administration, for detection of behaviour changes. After that period, animals were euthanised, weighted and, from each animal, heart, spleen, liver and kidneys were removed, weighted and observed for macroscopical changes. Relative organ weight was calculated as the ratio between the organ weight and the weight of the animal. The obtained results are presented from **Table 3** to **20.**

Table 3 – Absolute animal weight (empty liposomes)

Dose (mL/Kg)		Administration routes i.v. Males	i.v. Females	i.p. Males	i.p. Females
Control	average	3,91E+01	2,93E+01	3,69E+01	2,78E+01
	standard deviation	3,67E+00	1,61E+00	1,94E+00	1,02E+00
2	average	3,75E+01	2,92E+01		
	standard deviation	1,42E+00	1,99E+00		
5	average	3,80E+01	2,76E+01		
	standard deviation	2,73E+00	8,12E-01		
10	average	3,67E+01	2,92E+01	3,75E+01	2,73E+01
	standard deviation	9,15E-01	1,73E+00	1,45E+00	1,33E+00
20	average			3,55E+01	2,92E+01
	standard deviation			4,60E+00	2,91E+00
30	average			3,48E+01	2,94E+01
	standard deviation			1,52E+00	1,60E+00

Table 4 – Absolute heart weight (empty liposome)

Dose (mL/Kg)		Administration routes i.v. Males	i.v. Females	i.p. Males	i.p. Females
Control	average	1,70E-01	1,42E-01	2,07E-01	1,44E-01
	standard deviation	2,55E-02	5,20E-03	2,38E-02	2,13E-02
2	average	1,87E-01	1,55E-01		
	standard deviation	8,81E-03	1,67E-02		
5	average	1,85E-01	1,37E-01		
	standard deviation	1,95E-02	2,09E-02		
10	average	1,96E-01	1,50E-01	2,11E-01	1,33E-01
	standard deviation	3,68E-02	6,17E-03	3,63E-02	2,15E-02
20	average			2,15E-01	1,34E-01
	standard deviation			3,04E-02	1,07E-02
30	average			1,92E-01	1,49E-01
	standard deviation			1,18E-02	2,47E-02

Table 5 – Absolute liver weight (empty liposomes)

Dose (mL/Kg)		Administration routes i.v. Males	i.v. Females	i.p. Males	i.p. Females
Control	average	1,72E+00	1,31E+00	1,89E+00	1,07E+00
	standard deviation	2,08E-01	4,25E-02	2,07E-01	9,86E-02
2	average	1,72E+00	1,24E+00		
	standard deviation	7,32E-02	1,39E-01		
5	average	1,62E+00	1,12E+00		
	standard deviation	1,74E-01	8,91E-02		
10	average	1,68E+00	1,21E+00	1,96E+00	1,06E+00
	standard deviation	8,40E-02	1,40E-01	1,14E-01	7,88E-02
20	average			1,95E+00	1,20E+00
	standard deviation			1,12E-01	1,68E-01
30	average			1,58E+00	1,19E+00
	standard deviation			1,25E-01	1,40E-01

Table 6 – Absolute spleen weight (empty liposomes)

Dose (mL/Kg)		Administration routes i.v. Males	i.v. Females	i.p. Males	i.p. Females
Control	average	1,03E-01	9,46E-02	1,26E-01	1,04E-01
	standard deviation	1,08E-02	5,36E-02	2,51E-02	2,66E-02
2	average	1,10E-01	9,86E-02		
	standard deviation	1,51E-02	4,86E-02		
5	average	1,21E-01	7,60E-02		
	standard deviation	9,11E-03	2,53E-02		
10	average	1,16E-01	1,07E-01	1,06E-01	7,46E-02
	standard deviation	1,14E-02	2,55E-02	8,21E-03	2,36E-02
20	average			1,27E-01	1,01E-01
	standard deviation			1,88E-02	1,88E-02
30	average			1,08E-01	8,97E-02
	standard deviation			8,35E-03	1,86E-02

Table 7 – Absolute kidneys weight (empty liposomes)

Dose (mL/Kg)		Administration routes i.v. Males	i.v. Females	i.p. Males	i.p. Females
Control	average	6,37E-01	3,64E-01	6,22E-01	3,11E-01
	standard deviation	7,83E-02	2,62E-02	5,57E-02	4,00E-02
2	average	6,13E-01	3,63E-01		
	standard deviation	6,42E-02	2,76E-02		
5	average	6,46E-01	3,20E-01		
	standard deviation	1,11E-01	4,23E-02		
10	average	6,63E-01	3,99E-01	6,02E-01	2,95E-01
	standard deviation	6,17E-02	4,82E-02	6,61E-02	2,20E-02
20	average			5,24E-01	3,00E-01
	standard deviation			2,62E-01	1,80E-02
30	average			6,29E-01	3,17E-01
	standard deviation			6,37E-02	2,64E-02

Table 8 – Absolute animal weight (TFL containing liposomes)

Dose (mL/Kg)		Administration routes i.v. Males	i.v. Females	i.p. Males	i.p. Females
Control	average	2,61E+01	2,06E+01	2,64E+01	1,96E+01
	standard deviation	1,42E+00	1,13E+00	1,82E+00	1,93E+00
2	average	2,52E+01	1,89E+01		
	standard deviation	7,53E-01	1,28E+00		
5	average	2,58E+01	1,97E+01		
	standard deviation	1,99E+00	7,31E-01		
10	average	2,62E+01	2,01E+01	2,45E+01	2,05E+01
	standard deviation	1,32E+00	9,32E-01	1,79E+00	1,60E+00
20	average			2,71E+01	2,07E+01
	standard deviation			2,32E+00	6,96E-01
30	average			1,96E+01	2,02E+01
	standard deviation			1,93E+00	2,67E+00

Table 9 – Absolute heart weight (TFL containing liposomes)

Dose (mL/Kg)		Administration routes: i.v. Males	i.v. Females	i.p. Males	i.p. Females
Control	average	1,43E-01	1,08E-01	1,38E-01	9,60E-02
	standard deviation	1,28E-02	1,10E-02	1,92E-02	8,98E-03
2	average	1,39E-01	1,05E-01		
	standard deviation	9,49E-03	1,18E-02		
5	average	1,37E-01	1,14E-01		
	standard deviation	7,03E-03	8,61E-03		
10	average	1,54E-01	1,04E-01	1,30E-01	9,65E-02
	standard deviation	9,80E-03	1,48E-02	1,56E-02	9,46E-03
20	average			1,57E-01	1,05E-01
	standard deviation			3,50E-02	5,74E-03
30	average			1,34E-01	9,62E-02
	standard deviation			1,55E-02	1,17E-02

Table 10 – Absolute liver weight (TFL containing liposomes)

Dose (mL/Kg)		Administration routes: i.v. Males	i.v. Females	i.p. Males	i.p. Females
Control	average	1,32E+00	9,71E-01	1,19E+00	8,80E-01
	standard deviation	5,16E-02	8,62E-02	7,82E-02	1,10E-01
2	average	1,20E+00	9,63E-01		
	standard deviation	1,21E-01	8,88E-02		
5	average	1,31E+00	9,79E-01		
	standard deviation	1,76E-01	2,86E-02		
10	average	1,17E+00	1,00E+00	1,21E+00	1,01E+00
	standard deviation	6,32E-01	5,03E-02	1,40E-01	8,45E-02
20	average			1,40E+00	9,97E-01
	standard deviation			1,30E-01	6,59E-02
30	average			1,27E+00	9,19E-01
	standard deviation			1,48E-01	1,73E-01

Table 11 – Absolute spleen weight (TFL containing liposomes)

Dose (mL/Kg)		Administration routes i.v. Males	i.v. Females	i.p. Males	i.p. Females
Control	average	9,02E-02	8,52E-02	8,25E-02	7,66E-02
	standard deviation	5,55E-03	8,24E-03	3,97E-03	1,75E-02
2	average	8,65E-02	7,98E-02		
	standard deviation	1,28E-02	8,21E-03		
5	average	9,47E-02	9,41E-02		
	standard deviation	2,51E-02	4,85E-03		
10	average	1,02E-01	9,16E-02	9,52E-02	9,05E-02
	standard deviation	2,24E-02	6,22E-03	7,56E-03	8,78E-03
20	average			9,45E-02	9,65E-02
	standard deviation			8,13E-03	5,22E-03
30	average			1,10E-01	8,72E-02
	standard deviation			4,03E-02	1,58E-02

Table 12 – Absolute kidneys weight (TFL containing liposomes)

Dose (mL/Kg)		Administration routes i.v. Males	i.v. Females	i.p. Males	i.p. Females
Control	average	3,86E-01	2,32E-01	3,56E-01	2,21E-01
	standard deviation	3,53E-02	2,41E-02	2,80E-02	3,16E-02
2	average	3,52E-01	2,13E-01		
	standard deviation	1,30E-02	1,98E-02		
5	average	3,62E-01	2,15E-01		
	standard deviation	4,37E-02	4,90E-02		
10	average	3,90E-01	2,25E-01	3,36E-01	2,17E-01
	standard deviation	2,35E-02	1,92E-02	3,23E-02	2,46E-02
20	average			3,61E-01	2,38E-01
	standard deviation			4,23E-02	1,88E-02
30	average			3,48E-01	2,14E-01
	standard deviation			2,94E-02	1,76E-02

Table 13 – Relative heart weight (empty liposomes)

Dose (mL/Kg)		Administration routes i.v. Males	i.v. Females	i.p. Males	i.p. Females
Control	average	4,35E-03	4,88E-03	5,61E-03	5,18E-03
	standard deviation	4,87E-04	3,60E-04	7,34E-04	7,66E-04
2	average	4,99E-03	5,31E-03		
	standard deviation	1,62E-04	2,77E-04		
5	average	4,88E-03	4,96E-03		
	standard deviation	5,63E-04	8,06E-04		
10	average	4,88E-03	5,16E-03	5,61E-03	4,91E-03
	standard deviation	3,60E-04	3,89E-04	9,03E-04	8,90E-04
20	average			6,08E-03	4,61E-03
	standard deviation			8,16E-04	4,65E-04
30	average			5,52E-03	5,07E-03
	standard deviation			2,82E-04	8,75E-04

Table 14 – Relative liver weight (empty liposomes)

Dose (mL/Kg)		Administration routes i.v. Males	i.v. Females	i.p. Males	i.p. Females
Control	average	4,39E-02	4,48E-02	5,12E-02	3,87E-02
	standard deviation	1,45E-03	2,38E-03	4,75E-03	3,30E-03
2	average	4,58E-02	4,27E-02		
	standard deviation	2,02E-03	2,84E-03		
5	average	4,27E-02	4,07E-02		
	standard deviation	3,10E-03	2,43E-03		
10	average	4,58E-02	4,14E-02	5,24E-02	3,86E-02
	standard deviation	2,98E-03	3,03E-03	3,36E-03	1,43E-03
20	average			5,59E-02	4,09E-02
	standard deviation			9,62E-03	3,32E-03
30	average			4,53E-02	4,04E-02
	standard deviation			2,71E-03	4,22E-03

Table 15 – Relative spleen weight (empty liposomes)

Dose (mL/Kg)		Administration routes: i.v. Males	i.v. Females	i.p. Males	i.p. Females
Control	average	2,65E-03	3,29E-03	3,42E-03	3,75E-03
	standard deviation	3,51E-04	1,95E-03	6,65E-04	9,22E-04
2	average	2,94E-03	3,33E-03		
	standard deviation	4,13E-04	1,59E-03		
5	average	3,19E-03	2,75E-03		
	standard deviation	2,49E-05	9,28E-04		
10	average	3,18E-03	3,67E-03	2,81E-03	2,74E-03
	standard deviation	3,57E-04	7,30E-04	1,26E-04	8,96E-04
20	average			3,66E-03	3,46E-03
	standard deviation			9,83E-04	4,51E-04
30	average			3,11E-03	3,05E-03
	standard deviation			1,42E-04	6,26E-04

Table 16 – Relative kidneys weight (empty liposomes)

Dose (mL/Kg)		Administration routes: i.v. Males	i.v. Females	i.p. Males	i.p. Females
Control	average	1,63E-02	1,24E-02	1,69E-02	1,12E-02
	standard deviation	1,36E-03	4,49E-04	1,81E-03	1,12E-03
2	average	1,63E-02	1,25E-02		
	standard deviation	1,27E-03	3,47E-04		
5	average	1,69E-02	1,16E-02		
	standard deviation	2,22E-03	1,64E-03		
10	average	1,81E-02	1,37E-02	1,60E-02	1,08E-02
	standard deviation	1,79E-03	1,53E-03	1,50E-03	1,16E-03
20	average			1,52E-02	1,03E-02
	standard deviation			8,41E-03	6,26E-04
30	average			1,81E-02	1,08E-02
	standard deviation			1,51E-03	1,10E-03

Table 17 – Relative heart weight (TFL containing liposomes)

Dose (mL/Kg)		Administration routes: i.v. Males	i.v. Females	i.p. Males	i.p. Females
Control	average	5,49E-03	5,27E-03	5,25E-03	4,90E-03
	standard deviation	4,52E-04	5,20E-04	7,39E-04	1,97E-04
2	average	5,50E-03	5,56E-03		
	standard deviation	4,97E-04	7,85E-04		
5	average	5,35E-03	5,79E-03		
	standard deviation	5,73E-04	5,42E-04		
10	average	5,90E-03	5,20E-03	5,28E-03	4,70E-03
	standard deviation	5,87E-04	7,05E-04	3,02E-04	2,23E-04
20	average			5,77E-03	5,07E-03
	standard deviation			1,04E-03	2,44E-04
30	average			5,19E-03	4,79E-03
	standard deviation			4,46E-04	3,87E-04

Table 18 – Relative liver weight (TFL containing liposomes)

Dose (mL/Kg)		Administration routes: i.v. Males	i.v. Females	i.p. Males	i.p. Females
Control	average	5,07E-02	4,72E-02	4,50E-02	4,48E-02
	standard deviation	3,17E-03	3,28E-03	2,38E-03	1,82E-03
2	average	4,77E-02	5,09E-02		
	standard deviation	4,03E-03	3,25E-03		
5	average	5,06E-02	4,98E-02		
	standard deviation	4,96E-03	1,78E-03		
10	average	4,43E-02	4,99E-02	4,93E-02	4,93E-02
	standard deviation	2,34E-02	2,75E-03	4,63E-03	1,43E-03
20	average			5,15E-02	4,81E-02
	standard deviation			1,31E-03	1,96E-03
30	average			4,93E-02	4,53E-02
	standard deviation			3,60E-03	2,70E-03

Table 19 – Relative spleen weight (TFL containing liposomes)

Dose (mL/Kg)		*Administration routes* i.v. Males	i.v. Females	i.p. Males	i.p. Females
Control	average	3,47E-03	4,15E-03	3,13E-03	3,87E-03
	standard deviation	2,14E-04	3,94E-04	1,04E-04	6,85E-04
2	average	3,42E-03	4,22E-03		
	standard deviation	4,35E-04	3,83E-04		
5	average	3,65E-03	4,78E-03		
	standard deviation	8,45E-04	1,91E-04		
10	average	3,86E-03	4,57E-03	3,90E-03	4,41E-03
	standard deviation	6,87E-04	2,34E-04	4,63E-04	1,18E-04
20	average			3,49E-03	4,66E-03
	standard deviation			2,25E-04	2,21E-04
30	average			4,24E-03	4,32E-03
	standard deviation			1,33E-03	4,76E-04

Table 20 – Relative kidneys weight (TFL containing liposomes)

Dose (mL/Kg)		*Administration routes* i.v. Males	i.v. Females	i.p. Males	i.p. Females
Control	average	1,48E-02	1,13E-02	1,35E-02	1,12E-02
	standard deviation	1,28E-03	5,42E-04	1,03E-03	5,59E-04
2	average	1,40E-02	1,12E-02		
	standard deviation	8,36E-04	5,44E-04		
5	average	1,40E-02	1,09E-02		
	standard deviation	1,19E-03	2,57E-03		
10	average	1,49E-02	1,12E-02	1,37E-02	1,06E-02
	standard deviation	6,29E-04	6,70E-04	6,60E-04	7,15E-04
20	average			1,33E-02	1,15E-02
	standard deviation			8,20E-04	5,48E-04
30	average			1,35E-02	1,07E-02
	standard deviation			8,32E-04	7,50E-04

The obtained values were statistically treated for the significance of variations (p=0,005). Obtained results for absolute weights (full animal and separate organs) in both formulations were compared between themselves and with absolute weight of control group. The total absence of toxicity of any of the injected formulations was concluded from the statistical analysis.

Biological activity evaluation

In order to evaluate the biological activity of TFL liposomal formulations prepared according to the present invention, one animal model of leishmaniasis was selected. BALB/c mice were infected with 2 x 10^7 (i.v.) LV-9 (*Leishmania donovani*) parasites, obtained from the London School of Hygiene and Tropical Medicine. The groups (5 animals per group) and treatment schedules are presented in **Table 21.**

Table 21 – Biological activity

Formulation lipidic composition	Dose (mg TFL/kg)	N^{er} of Doses	Inhibition %
DOPC:DOPG 7:3	15	5 3 1	62 17 80
DSPC:CHOL 4:1	15	5 3 1	53 92 91
PC:PG 4:1	15	5 3 1	47 88 60
PC:CHOL:PI 3.7:1:0,3	5	5 3 1	57 52 68
PC:CHOL:DSPE-PEG (2000) 3.7:1:0,3	4	5 3	75 74
DOPC:DOPG 7:3 (dialysed)	0,6	5	86

Treatments started 7 days post-infection and animals were euthanised 15 days post-infection. Liver was removed and weighted from each animal and amastigote counting was performed in each one by smear impression. The infection was calculated through an appropriate mathematical equation. The so obtained results are expressed in **Table 21**.

The first conclusion is that, due to the liposomal incorporation of TFL, parenteric administration of TFL is possible.

All TFL liposomal formulations were able to reduce infection in this model.

Claims

1. A liposomal formulation characterized by the fact of containing one dinitroaniline incorporated or encapsulated.

2. A liposomal formulation, according to claim 1, characterized by the fact of the dinitroaniline being trifluralin.

3. A liposomal formulation, according to any of the claims 1 and 2, characterized by the fact of containing liposomes with diameter varying from 0.01 µm to 50 µm.

4. A liposomal formulation, according to any of the claims 1 to 3, characterized by the fact of mixing populations of particles with different diameter.

5. A liposomal formulation, according to any of the claims 1 to 4, characterized by the fact of mixing populations of particles, respectively bigger and lower than 100 nm.

6. Liposomal formulations, according to any of the previous claims, characterized by the fact of being prepared with any of the following lipids, hydrogenated or not, individually or in mixtures, in any molar ratio: distearoylphosphatidylcholine (DSPC), phosphatidylcholine (PC), cholesterol (Chol) or derivatives, sphingomielin (SM), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), phosphatidylglycerol (PG), dimiristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), gangliosides, ceramides, phosphatidylinositol (PI), phosphatydic acid (PA), dicetylphosphate (DcP), dimiristoylphosphatidylglycerol, (DMPG), stearylamine (SA), dipalmitoylphosphatidylglycerol (DPPG) and other synthethic lipids.

7. Process for the preparation of a liposomal formulation containing one dinitroaniline, characterized by:

- obtention of a liposomal preparation containing a dinitroaniline by hydration of a lipidic film containing the dinitroaniline
- lyophilization of the dinitroaniline liposomal formulation
- rehydration of the dehydrated liposomal formulation

8. Process according to claim 7, characterized by the performing of a sizing step of the dinitroaniline liposomal formulation in order to reduce the vesicle diameter, done previously to the dehydration step.

9. Process, according to claim 8, characterized by the performing of the sizing step by extrusion under pressure through porous membranes.

10. Process, according to any of the claims 7 to 9, characterized by the fact that the hydration is carried out by the addition of a small amount of an aqueous solution, followed by the addition of the remaining volume of the aqueous solution, after a resting period.

11. Process, according to claim 10, characterized by the fact of using, in the hydration steps, a non-saline solution.

12. Process, according to claim 11, characterized by the fact of performing the rehydration steps with saccharose, trehalose, glucose or any other sugar solution.

13. Process, according to any of the claims 7 to 12, characterized by the fact of mixing different diameter particle populations.

14. Process, according to claim 13, characterized by the fact of mixing particles that, after sizing, present population with diameters of, respectively, bigger and lower than 100 nm.

15. Process, according to claim 14, characterized by the fact of performing the sizing step according to claim 9.

16. Process, according to any of the claims 7 to 9 or 13 to 15, characterized by the fact that the hydration is performed according to claim 10.

17. Process, according to any of the claims 7 to 10 or 13 to 16, characterized by the fact of using in the hydration step a solution according to claim 11.

18. Process, according to any of the claims 7 to 11 or 13 to 17, characterized by the fact of using solutions according to claim 12.

19. Process, according to any of the claims 7 to 18, characterized by the use of any of the following lipids, hydrogenated or not, individually or in mixtures, in any molar ratio: distearoylphosphatidylcholine (DSPC), phosphatidylcholine (PC), cholesterol (Chol) or derivatives, sphingomielin (SM), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), phosphatidylglycerol (PG), dimiristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), gangliosides, ceramides, phosphatidylinositol (PI), phosphatydic acid (PA), dicetylphosphate (DcP), dimiristoylphosphatidylglycerol, (DMPG), stearylamine (SA), dipalmitoylphosphatidylglycerol (DPPG) and other synthethic lipids.

20. Process, according to any of the claims 7 to 19, characterized by the fact of the dinitroaniline is trifluralin.

21. Process, according to any of the claims 1 to 6, when prepared by a process according to any of the claims 7 to 20.

22. Use of the liposomal formulations for the treatment in humans or animals, characterized by the use of a therapeutic efficient quantity of a dinitroaniline liposomal formulation according to any of the claims 1 to 6 and 21.

Effect of the presence of cholesterol

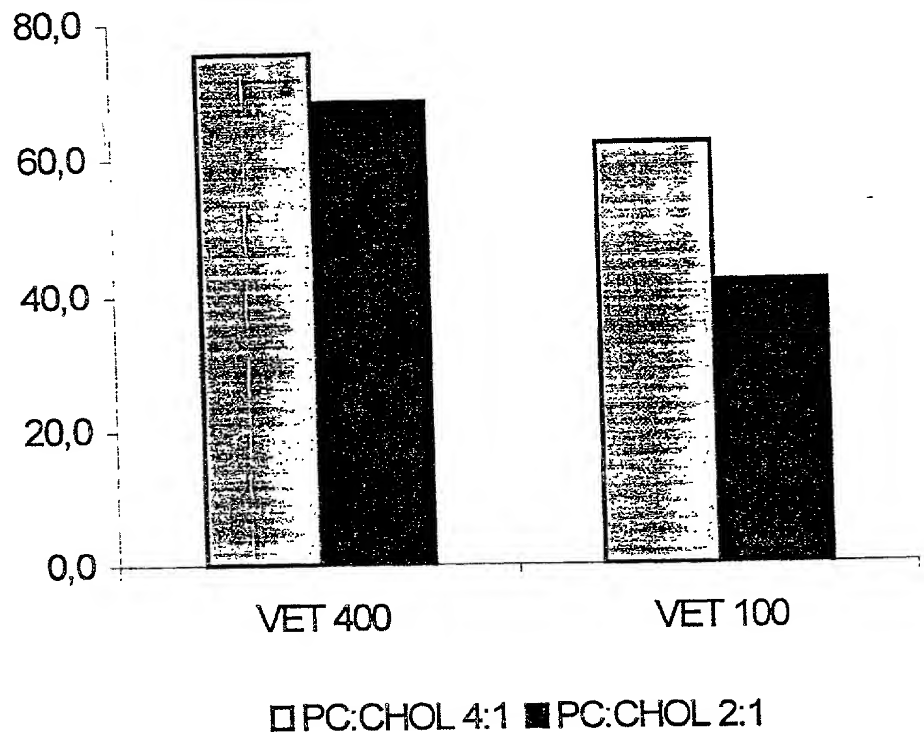

Figure 1

Effect of the presence of electric charge in the lipid membrane

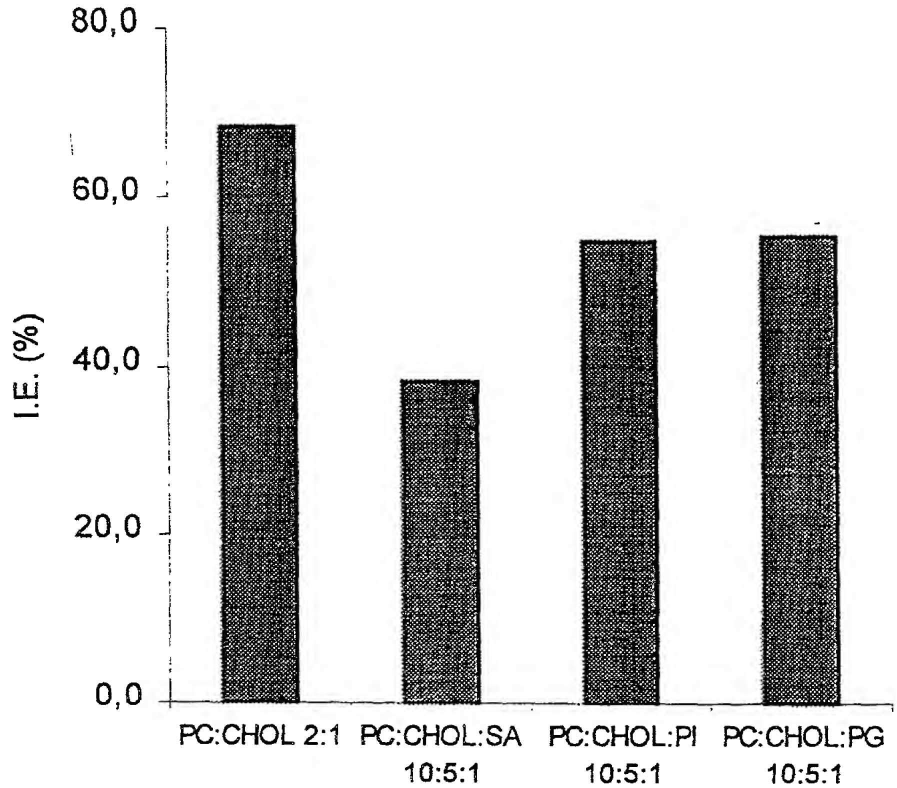

Figure 2

Effect of transition phase temperature of lipids

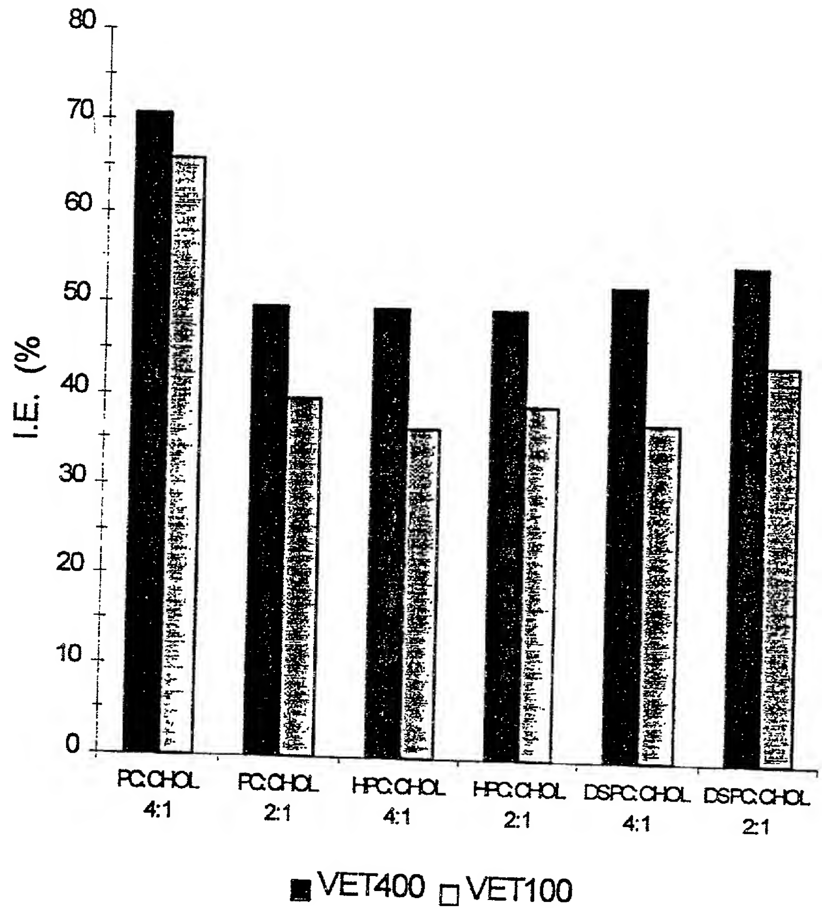

Figure 3

In vitro stability of two formulations of PC:PG 4:1

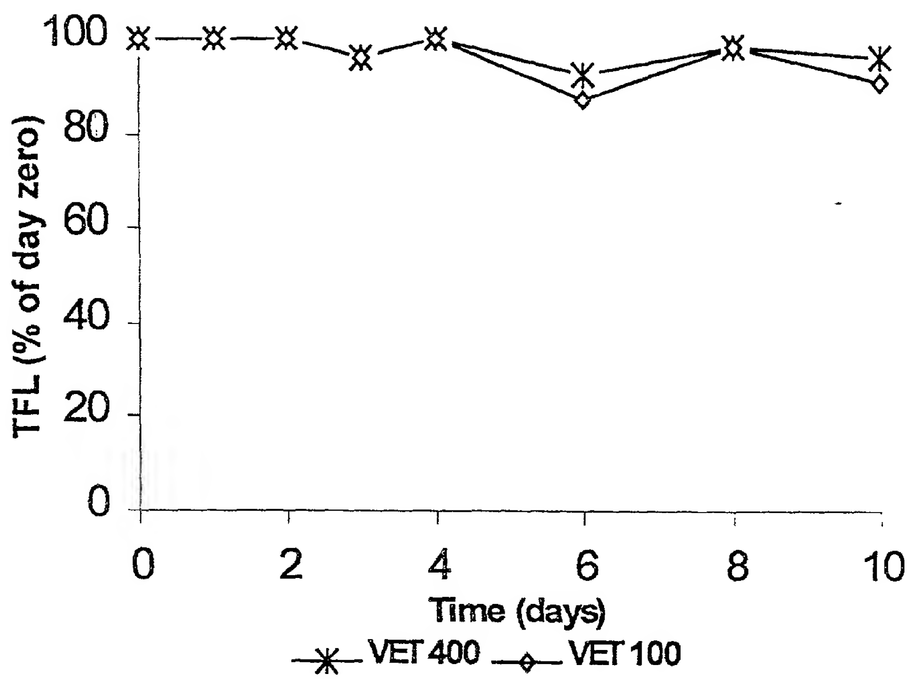

Figure 4

09/529937

Stability on storage of three liposomal formulations of DOPC:DOPG 7:3

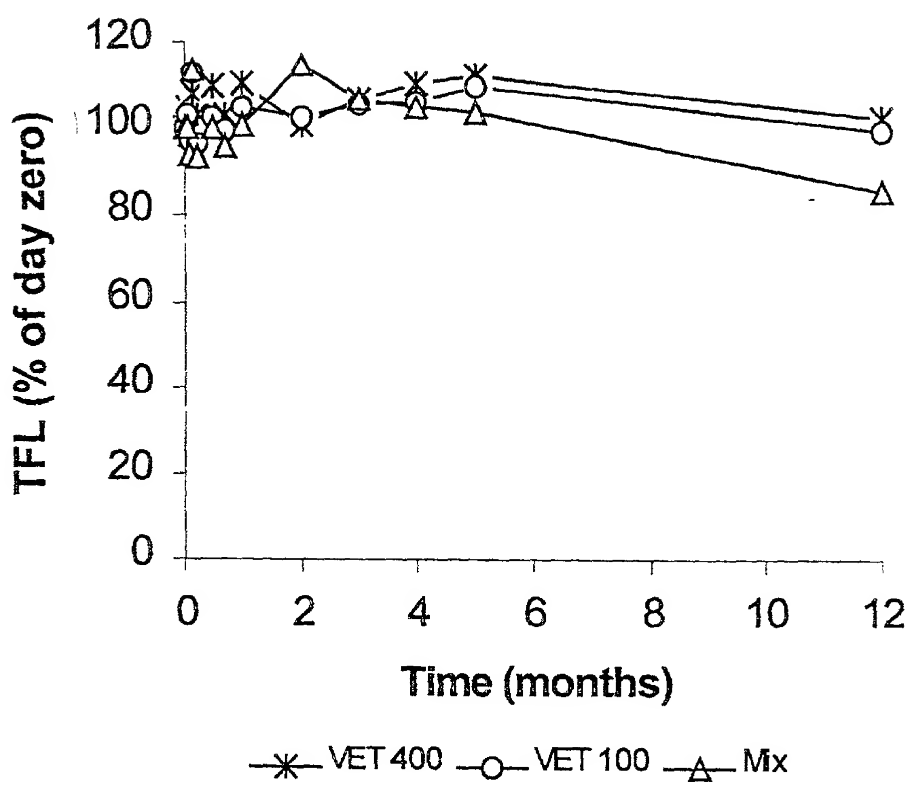

Figure 5

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COMBINED DECLARATION AND POWER OF ATTORNEY FOR PATENT AND DESIGN APPLICATIONS

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated next to my name; that I verily believe that I am the original, first and sole inventor (if only one inventor is named below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Insert Title: DINITROANILINE LIPOSOMAL FORMULATIONS AND PROCESSES FOR THEIR PREPARATION

Fill in Appropriate Information - For Use Without Specification Attached:

the specification of which is attached hereto. If not attached hereto,
the specification was filed on April 21, 2000 as
United States Application Number 09/529,937;
and amended on ____ (if applicable) and/or
the specification was filed on ____ as PCT
International Application Number PCT/PT99/00015; and was
amended under PCT Article 19 on ____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I do not know and do not believe the same was ever known or used in the United States of America before my or our invention thereof, or patented or described in any printed publication in any country before my or our invention thereof or more than one year prior to this application, that the same was not in public use or on sale in the United States of America more than one year prior to this application, that the invention has not been patented or made the subject of an inventor's certificate issued before the date of this application in any country foreign to the United States of America on an application filed by me or my legal representative or assigns more than twelve months (six months for designs) prior to this application, and that no application for patent or inventor's certificate on this invention has been filed in any country foreign to the United States of America prior to this application by me or my legal representatives or assigns, except as follows.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Insert Priority Information: (if appropriate)

Prior Foreign Application(s)			Priority Claimed	
102197 (Number)	Portugal (Country)	August 21, 1998 (Month/Day/Year Filed)	☒ Yes	☐ No
(Number)	(Country)	(Month/Day/Year Filed)	☐ Yes	☐ No
(Number)	(Country)	(Month/Day/Year Filed)	☐ Yes	☐ No
(Number)	(Country)	(Month/Day/Year Filed)	☐ Yes	☐ No

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional applications(s) listed below.

Insert Provisional Application(s): (if any)

(Application Number)	(Filing Date)
(Application Number)	(Filing Date)

All Foreign Applications, if any, for any Patent or Inventor's Certificate Filed More than 12 Months (6 Months for Designs) Prior to the Filing Date of This Application:

Insert Requested Information: (if appropriate)

Country	Application Number	Date of Filing (Month/Day/Year)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States and/or PCT application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States and/or PCT application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is material to the patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

Insert Prior U.S. Application(s): (if any)

(Application Number)	(Filing Date)	(Status - patented, pending, abandoned)
(Application Number)	(Filing Date)	(Status - patented, pending, abandoned)

I hereby appoint the following attorneys to prosecute this application and/or an international application based on this application and to transact all business in the Patent and Trademark Office connected therewith and in connection with the resulting patent based on instructions received from the entity who first sent the application papers to the attorneys identified below, unless the inventor(s) or assignee provides said attorneys with a written notice to the contrary:

17

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Residence (City, State & Country)		CITIZENSHIP
POST OFFICE ADDRESS (Complete Street Address including City, State & Country)		

*DATE OF SIGNATURE